Quartz-Crystal Microbalance Immunosensor for *Schistosoma-japonicum*-Infected Rabbit Serum

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A quartz-crystal microbalance immunosensor (QCM) has been developed for the direct determination of *Schistosoma-japonicum*-infected rabbit serum. A self-assembled monolayer with carboxyl groups was first coated on a gold electrode of a quartz-crystal resonator by the spontaneous adsorption of 3-mercaptopropionic acid. *Schistosoma-japonicum* molecular antigen of 32 kD molecular weight was then covalently attached to the crystal surface. The QCM immunosensor was used to detect infected rabbit serum (IRS49-2000); a maximum titer of 1:800 was achieved.

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Schistosomiasis is a tropical parasitic disease which infects numerous and scattered population. The classical diagnosis method for the disease is to detect antibodies in serum using an enzyme-linked immunosorbent assay (ELISA).¹ The method, however, requires additional labels, and it is difficult to realize digitization and to build a portable device. The development of a simple and effective transducer device for the direct determination of *Schistosoma-japonicum* antibody (SjAb) deserves further investigation.

A piezoelectric quartz-crystal microbalance (QCM) as a versatile class of sensor is highly sensitive to mass changes on crystal surfaces. Many applications have been found in chemical and environmental fields.^{2,3} Recently, some efforts have been directed to the development of QCM immunosensors for clinical immunoassay.^{4,5} The general approach to utilize a piezoelectric device as an immunosensor is to coat the crystal with antibodies or antigens. Complementary proteins, when binding to immobilized antibodies or antigens, can cause a resonance frequency change of the quartz crystal. Since the QCM immunosensors respond directly to the mass of antibodies or antigens, they do not require any additional labels, and we can on-line monitor the immunoreactions.

For QCM immunosensors, techniques for depositing thin organic films on quartz-crystal surfaces play an important role in the immobilization of antibodies or antigens. The quality of films has great effects on the sensitivity, reproducibility, and detection limit of sensors. The conventional method is to coat a polymer film capable of forming hydrophobic and/or covalent bonds with antibodies.⁶ These polymer films often suffer from several disadvantages. First, polymers are usually adsorbed to the crystal surfaces only by hydrophobic bonds. Since hydrophobic bonds are not quite firm, they can be broken by a mechanical interaction in washing and detecting procedures, or a pH shift of a solution. Therefore, these polymer films can be easily separated from the quartz-crystal surfaces. This may

result in a decrease in the reproducibility and sensitivity of the immunosensor. Second, the swelling of polymer films in aqueous solution may cause the lose of part of the sensitivity and stability of the piezoelectric immunosensor. A silane layer is another useful coating,^{7,8} however, because it can be integrated with the quartz crystal, the quartz-crystal resonator can not be used repeatedly.

Self-assembled monolayer (SAM) techniques^{9,10} offer a new strategy for the immobilization of antibodies or antigens. Since surfur donor atoms strongly coordinate on metal substrates, such as Au, Ag, Pt *etc.*, surfur-containing molecules, such as disulfides (R-SS-R), sulfides (R-S-R) and thiols, can form various functionalized self-assembled monolayers, which are of highly organized and compact construction. Besides good mechanical and chemical properties, an additional advantage of the self-assembled monolayers is that they make it feasible to design the crystal surface at the molecular level for the immobilization of bio-molecules, such as proteins and DNAs. Some applications of the SAM techniques in the immobilization of bio-molecules have been reported. Knoll and co-workers¹¹ presented a versatile and useful biotin-functionalized SAM. When avidins are bound to the monolayer, biotinylated antibodies are readily immobilized. Schlereth¹² reported surface-modified gold electrodes with biospecific affinity for NAD(H)-dependent lactate dehydrogenase (LDH) using mixed self-assembled monolayers bearing coenzyme-analogous, Cibacron Blue 3FG-A, as an anchored pendant group. Enzymatic monolayers are produced by the formation of a complex upon the site-specific binding of Cibacron Blue to the enzyme through its NAD⁺-binding packet. Caruso *et al.*¹³ reported on the immobilization of an anti-IgG antibody using a monolayer of 3-mercaptopropionic acid (MPA) for immunosensing.

This paper reports on a QCM immunosensor for the direct detection of *Schistosoma-japonicum*-infected rabbit serum (Sj-IRS). A *Schistosoma-japonicum* antigen with 32 kD of molecular weight (SjAg32) is covalently immobilized onto the quartz-crystal surface *via* a self-assembled monolayer with

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carboxyl groups. The QCM immunosensor is applied for the determinations of infected rabbit serum (IRS49-2000) and several rabbit serum samples with different degrees of infection.

Experimental

Apparatus

The quartz-crystal resonators used (Beijing Chenxing Radio Equipments) were 9 MHz, AT-cut quartz wafers with vacuum-deposited gold electrodes on both sides. A homemade transistor-transistor logic integrated circuit (TTL-IC) was designed to drive the quartz crystal at its resonant frequency. The resonant frequency was monitored with a frequency counter (Model FC1250 Wellstar). An HP 4395 A network/impedance analyzer was employed to perform admittance measurements on the quartz crystal. A Model CSS501 thermostat (Chongqing Experimental Equipment Factory) controlled the incubating temperature.

Materials

A schistosoma japonium molecular antigen of 32 KD molecular weight (SjAg32) was isolated from an adult worm antigen (AWA) and purified to homogeneity according to a reported method.¹⁴ Infected rabbit serum (IRS49-2000) was prepared by immunizing rabbits for 49 days with 2000 schistosoma japonium, as described elsewhere.¹⁵ Normal rabbit serum (NRS) was obtained by mixing the sera of three normal rabbits. 3-Mercaptopropionic acid (MPA) was purchased from Fluka. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were obtained from Sigma. The HEMA/MMA copolymer was prepared by copolymerizing hydroxyethyl methacrylate (HEMA) and methyl methacrylate (MMA) in this laboratory, as described elsewhere.¹⁶ All other reagents and solvents were of analytical-reagent grade, and double distilled water was used throughout.

Immobilization procedure

The immobilization of SjAg32 was performed stepwise. Initially, quartz crystals were pretreated as described elsewhere.¹⁷ They were exposed to a piranha solution consisting of one part 30% H₂O₂ in three parts H₂SO₄ for 3 min, followed by rinsing thoroughly with distilled water. This process was repeated twice. Since piranha solution is an extremely strong oxidant, caution must be taken and only small volumes should be prepared at one time. In all cases, the quartz crystals were used immediately after this treatment. The crystals were then exposed to an MPA aqueous solution for 12 h, washed with distilled water and dried under a nitrogen stream. Self-assembled monolayers of 3-mercaptopropionic acid (MPA-SAM) were thus formed on the crystal surfaces. The MPA-coated crystals were activated with an EDC/NHS mixture solution (containing 5 mg/ml EDC and 5 mg/ml NHS) for 30 min. After washing with distilled water and drying under a nitrogen stream, 10 μ l of SjAg32 solution in a phosphate buffer (PB) of pH 7.4 was then added to the activated crystal surfaces, and incubated at 37°C in a 100% humidity environment. After 60 min, the SjAg32 were covalently bonded to the crystal surfaces. A bovine serum albumin (BSA) solution of 10 mg/ml in PB of pH 7.4 was then added to the SjAg32-modified crystal surfaces for 30 min to block the binding sites for proteins. Finally, the crystals were washed with phosphate buffered saline (PBS) of pH 7.4, followed by thoroughly washing with double-distilled water.

Fig. 1 Conductance-frequency spectra of the quartz crystal. (a) The bare crystal; (b) after coating SAM; (c) after coating HEMA-MMA copolymer film.

Measurement procedure

The fundamental frequency (F_1) of the SjAg32-modified crystals were monitored first in a drying chamber. The crystals were then exposed to IRS solutions of different concentrations, diluted with PBS (pH 7.4), and incubated at 37°C for 60 min. The crystals were successively washed with PBS and distilled water. The resonant frequencies were monitored in a drying chamber, and then the frequency shifts (ΔF) were obtained. As a control, the same concentration of NRS was incubated under the same conditions in each experiment.

Regeneration of quartz crystals

At the end of each assay, the crystals were washed with the piranha solution for 3 min three times to remove the self-assembled monolayer and proteins on the crystal surfaces. After such treatments, the resonance frequencies of the quartz crystals were returned, and the quartz crystals could be repeatedly used.

Discussion

Performance of a MPA-coated crystal

The MPA-SAM formed by the spontaneous adsorption of MPA molecules is bound to the gold electrode surfaces through robust S-Au bonds.^{9,10} It has good mechanical and chemical properties. Caruso *et al.*¹³ demonstrated the successful application of the MPA-SAM in the immobilization of an anti-IgG antibody. Here, the oscillation behavior of the MPA-coated crystal was investigated. Figure 1 shows the conductance-frequency spectra of the quartz crystal. When coating an HEMA-MMA copolymer film, besides a significant decrease in the frequency of the maximum conductance ($f_{G_{max}}$), a clear decrease in the maximum conductance (G_{max}) and a broadening of the bandwidth of the filter ($\Delta f_{1/2}$) at the half-power points on the conductance-frequency spectra are observed. Here, the $\Delta f_{1/2}$ is equal to the peak-width-at-half-height of the conductance-frequency spectra. This indicates that the polymer membrane is a viscous film and that part of the mass sensitivity of the quartz crystal will be lost. When assembling an MPA monolayer on a crystal surface, however, only a slight decrease in G_{max} and a small broadening of the bandwidth of the filter emerged,

Fig. 2 Effects of the SjAg concentration on the amount of bound SjAg and on the response to the IRS49-2000 of 1 to 10 dilution.

suggesting that the MPA monolayer on the crystal surface has little effect on the oscillating performance of the crystal. According to Buttry,¹⁸ the bandwidth and the resonance frequency (f_s) determine the quality factor (Q), which can be expressed as

$$Q = f_s / \Delta f_{1/2} \approx f_{Gmax} / \Delta f_{1/2} \quad (2)$$

The Q values of a bare crystal, an MPA-coated crystal and a polymer-coated crystal were obtained from the conductance spectra; they are 3.19×10^4 , 3.17×10^4 and 2.52×10^4 , respectively. This result indicates that the MPA-coated crystal has a similar stable oscillation behavior as a bare crystal, while the oscillation stability of a polymer-coated crystal clearly decreases.

Effects of the SjAg concentration in the immobilization solution

The amount of bound antigens on the crystal surface has a great effect on the frequency response of the immunosensor. With a view to the mass sensitivity of the quartz crystal, more active antigens per unit area on the crystal surfaces allows more antibodies to be bound to the crystal, while producing a greater response of the QCM immunosensor. The effect of the concentration of SjAg in the immobilization solution on the amount of bound SjAg on the crystal surface and on the corresponding frequency response of the SjAg-modified crystal to the SjAb solution with a dilution of 1:10 (Fig. 2) was investigated. It was found that the frequency shift resulting from the immobilization of SjAg increases with the concentration of SjAg in the range of 0–2.0 mg ml⁻¹ (Fig. 2a). That is to say, more antigens are bound to the crystal with an increase in the SjAg concentration. The frequency shift due to the immune adsorption of SjAb also increases with an increase in the SjAg concentration in a range of a relatively low concentration (Fig. 2b). However, when the SjAg concentration becomes 0.4 mg ml⁻¹ or higher, the frequency-response value to SjAb tends not to increase any more. This seems to be related to the following fact. When the concentration of SjAg is in the low-concentration range, only a small number of SjAg are immobilized to the crystal. Because the effective active SjAg increases with the amount of bound SjAg, the SjAb absorbed through immunoreaction also increases. After the concentration of SjAg in the immobilization solution reaches 0.4 mg ml⁻¹ or higher, the number of bound SjAg still increase with the

Fig. 3 Dependency of the amount of bound SjAg on the incubation period.

concentration of SjAg in the immobilization solution, while the steric hindrance among SjAg molecules also keeps increasing, the effective active SjAg per area on the crystal surface does not increase any more, and the SjAb absorbed through immunoreactions tends not to increase. In this experiment, the SjAg of 1.0 mg ml⁻¹ was used as the immobilization solution, unless otherwise indicated.

Effect of the incubation time in the SjAg immobilizing process

The effect of the incubation time in the SjAg immobilizing process was investigated (Fig. 3). The antigens were covalently bound to the crystal through their amine residues, and the reaction was carried out quickly. Figure 3 shows that the amount of SjAg on the crystal increases with increasing the incubation period. The amount of SjAg after incubating for 40 min tended to be stable, and the optimum in the incubation period was reached after 60 min.

Standard curve and sample tests

To obtain the standard curve of the QCM immunosensor, IRS 49-2000 solutions with different dilutions were detected. As a control, the same dilutions of NRS sera were tested. As can be seen from Fig. 4, the frequency responses increased with the SjAb concentration in the dilution range of 1:800–1:10. A dilution of 1:800, with a frequency-response value of 29 Hz, which is larger than three-time standard deviation of control assays (± 6.2 Hz), is considered to be the maximum titer of IRS49-2000 with this QCM immunosensor. The detection errors are also shown in Fig. 4 in the form of error bars. For the 1:20 of IRS 49-2000 serum sample solution, the mean response value of five repeated tests was 144 ± 8.2 Hz, and the relative standard deviation was 5.7%. Furthermore, six IRS samples of different infection degrees were tested, which were prepared by infecting rabbits with different numbers of schistosoma japonicum and infection times. Table 1 gives the frequency responses of the QCM immunosensor to these samples with dilutions of 1:20. It can be indicated that the frequency shifts tend to increase with an increase in the infection degree. The immunosensor has large frequency responses to those IRS with serious infection degree prepared with much schistosoma japonicum for infecting rabbits and a long infection time, and has small frequency responses to these sera prepared with few schistosoma-japonicum and a short infection time. The result

Table 1 Frequency responses of rabbit serum samples with different degrees of infection

Rabbit serum	1	2	3	4	5	6
Degree of infection	28 ^a - 2000 ^b	45 - 1000	31 - 1000	180 - 100	35 - 2000	49 - 2000
Frequency shift/Hz	94 ± 6.4	137 ± 5.5	92 ± 7.2	151 ± 8.8	133 ± 6.8	144 ± 8.2

The infection degree is qualitatively expressed with the infecting days (a) and the number of *Schistosoma japonicum* (b) for the infection of rabbits.

Fig. 4 The standard curve of the QCM immunosensor for IRS49-2000.

implies that this QCM immunosensor can be used to determine the infection degree of Sj-IRS.

Regeneration of quartz crystals

After each experiment, a regeneration process is necessary. The SAM and adsorbed proteins on the crystal are easily rinsed out by a piranha solution. The change in the resonance frequency of the crystal after such a detection circuit is less than ± 40 Hz. The small change in the resonance frequency has no effect on the performance of the crystal. In this experiment, the crystal is repeatedly used for 50 cycles without any significant change in its performance.

Conclusion

The QCM immunosensor methods, as potential alternatives to conventional immunoassay, need no additional reagents, such as radiotracer, fluorescent or enzymatic agents. We achieved success in the immobilization of SjAg on QCM through an SAM of mercaptopropionic acid and in the use of the QCM immunosensor for the determination of Sj-IRS. The recognition of infected and normal rabbit sera and the determination of the degree infection were demonstrated. This approach seems to be

promising for further applications in the medical diagnostics for other parasites and viruses, and even bacteria.

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